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UTILITY PATENT APPLICATION TRANSMITTAL UNDER 37 CFR §1.53(b)	
Attorney Docket Number	00742/056003
Applicant	Junying Yuan, Alexei Degterev, Tim Mitchison
Title	SMALL MOLECULE INHIBITORS OF NECROSIS

PRIORITY INFORMATION:

This application claims priority from United States provisional patent applications serial nos. 60/159,668 and 60/174,749, filed October 15, 1999, and January 6, 2000, respectively.

APPLICATION ELEMENTS:

Cover sheet	1 pages
Specification	41 pages
Claims	18 pages
Abstract	1 page
Drawing	8 sheets
Combined Declaration and POA, which is: ☒ Unsigned; ☐ Newly signed for this application; ☐ A copy from prior application [**SERIAL NUMBER**] and the entire disclosure of the prior application is considered as being part of the disclosure of this new application and is hereby incorporated by reference therein.	3 pages
Statement Deleting Inventors	[**] pages
Sequence Statement	[**] pages
Sequence Listing on Paper	[**] pages
Sequence Listing on Diskette	[**] disk
Small Entity Statements, which are: ☐ Unsigned; ☐ Newly signed for this application; ☒ Copies from prior applications serial nos. 60/159,668 and 60/174,749 and such small entity status is still proper and desired.	2 pages
Preliminary Amendment	[**] pages

IDS	[**] pages
Form PTO 1449	[**] pages
Cited References	[**] references
Recordation Form Cover Sheet and Assignment	[**] pages
Assignee's Statement	[**] pages
English Translation	[**] pages
Certified Copy of Priority Document	[**] pages
Return Receipt Postcard	1
FILING FEES:	
Basic Filing Fee: $355	$355.00
Excess Claims Fee: 79 -20 = 59 x $9	$531.00
Excess Independent Claims Fee: 13 -3 = 10 $40	$400.00
Multiple Dependent Claims Fee: $135	$135.00
Total Fees:	$1,421.00

☒ Enclosed is a check for $1,421.00 to cover the total fees.
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K. Bieker-Brady
Signature

October 13, 2000
Date

ATTORNEY DOCKET NO. 00742/056002

Applicant or Patentee	: Junying Yuan *et al.*
Serial or Patent No.	: 60/174,749
Filed or Issued	: January 6, 2000
Title	: SMALL MOLECULE INHIBITORS OF NECROSIS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:
Name of Organization: President and Fellows of Harvard College
Address of Organization: 17 Quincy Street, Cambridge, MA 02138
Type of Organization:

[X] University or Other Institution of Higher Education
[ ] Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
[ ] Nonprofit Scientific or Educational under Statute of State of the United States of America
Name of State:
Citation of Statute:
[ ] Would Qualify as Tax Exempt under Internal Revenue Service Code (26 Usc 501(a) and 501(c)(3)) If Located in the United States of America
[ ] Would Qualify as Nonprofit Scientific or Educational under Statute of State of the United States of America If Located in the United States of America
Name of State:
Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled SMALL MOLECULE INHIBITORS OF NECROSIS by inventors Junying Yuan, Alexei Degterev, and Tim Mitchison described in

[ ] the specification filed herewith.
[X] application serial no. 60/174,749, filed January 6, 2000.
[ ] patent no. [**PATENT NUMBER**], issued [**ISSUE DATE**].

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name:
Address:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Jeffrey Labovitz, Ph.D.

Title: Director, Office of Technology Licensing

Address: Harvard Medical School, Office of Technology Licensing & Industry Sponsored Research, 25 Shattuck Street Building A, Room 414, Boston, MA 02115

Signature: ______ **Date:** 4/14/00

09/755145 - 011301

ATTORNEY DOCKET NO. 00742/056001

Applicant or Patentee	: Junying Yuan et al.
Serial or Patent No.	: 60/159,668
Filed or Issued	: October 15, 1999
Title	: SMALL MOLECULE INHIBITORS OF TNFα INDUCED NECROSIS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:
Name of Organization: President and Fellows of Harvard College
Address of Organization: 17 Quincy Street, Cambridge, MA 02138
Type of Organization:

[X] University or Other Institution of Higher Education
[ ] Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
[ ] Nonprofit Scientific or Educational under Statute of State of the United States of America
Name of State:
Citation of Statute:
[ ] Would Qualify as Tax Exempt under Internal Revenue Service Code (26 Usc 501(a) and 501(c)(3)) If Located in the United States of America
[ ] Would Qualify as Nonprofit Scientific or Educational under Statute of State of the United States of America If Located in the United States of America
Name of State:
Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled SMALL MOLECULE INHIBITORS OF TNFα INDUCED NECROSIS by inventors Junying Yuan, Alexei Degterev, and Tim Mitchison described in

[ ] the specification filed herewith.
[X] application serial no. 60/159,668, filed October 15, 1999.
[ ] patent no. [**PATENT NUMBER**], issued [**ISSUE DATE**].

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name:
Address:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Joyce Brinton

Title: Director, Office for Technology & Trademark Licensing

Address: 124 Mt. Auburn Street, Cambridge, MA 02139

Signature: _____ **Date:** 11/8/99

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Guy Beardsley
Printed name of person mailing correspondence

Signature of person mailing correspondence

APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANTS : JUNYING YUAN
ALEXEI DEGTEREV
TIM MITCHISON

TITLE : SMALL MOLECULE INHIBITORS OF NECROSIS

SMALL MOLECULE INHIBITORS OF NECROSIS

Cross Reference To Related Applications

This application claims priority from U.S. Provisional Application Serial Nos. 60/159,668, filed October 15, 1999 and 60/174,749, filed January 6, 2000.

Statement as to Federally Sponsored Research

This invention was made in part with Government funding, and the Government therefore has certain rights in the invention.

Background of the Invention

In general, the invention relates to methods and compounds used to decrease necrosis.

In many diseases, cell death is mediated through apoptotic and/or necrotic pathways. While much is known about the mechanisms of action that control apoptosis, control of necrosis is not as well understood. Understanding the mechanisms regulating both necrosis and apoptosis in cells is essential to being able to treat conditions, such as neurodegenerative diseases, stroke, coronary heart disease, kidney disease, and liver disease. A thorough understanding of necrotic and apoptotic cell death pathways is also crucial to treating AIDS and the conditions associated with AIDS, such as retinal necrosis.

Research has shown that caspases play a central role in the induction of apoptosis. Peptide based inhibitors of caspases, such as zVAD-fmk are useful in preventing activation of the apoptotic cell death pathway in cells stimulated to

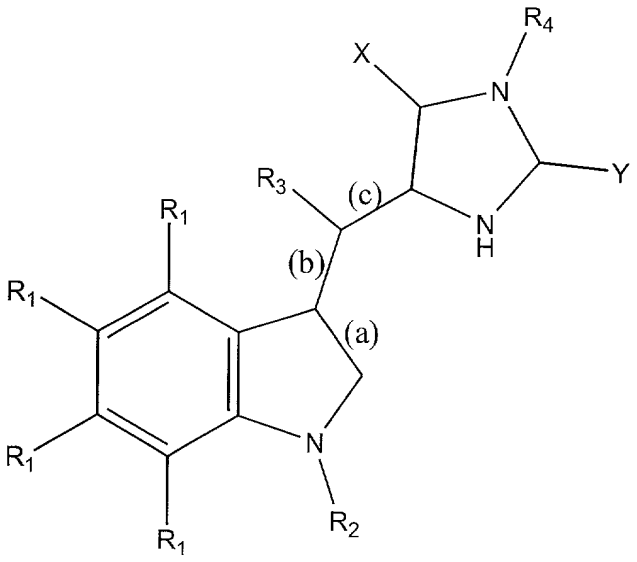
undergo apoptosis by compounds such as TNFα. However, cells treated with zVAD-fmk and these cell death stimuli still die through a caspase-independent form of necrosis.

Discovery of a compound which prevents caspase-independent cell death (necrosis) would provide a useful therapeutic for treating conditions in which necrosis occurs, and for preventing the onset of necrosis. These compounds and methods may be particularly useful for treating ischemic brain and heart injuries and head traumas.

Summary of the Invention

The present invention features methods and compounds for decreasing necrosis. The compounds of the present invention may be used as therapeutics to decrease necrosis in a desired cell, such as a neuron. These compounds are characterized by their ability to decrease necrosis in response to modulation of intracellular signaling pathways, such as those activated by TNFα. By also treating the cells with zVAD-fmk, we have inhibited the apoptotic pathway. Accordingly, we have been able to determine that the compounds of the invention specifically decrease necrosis. In addition, we have shown that the identified compounds that decrease necrosis in response to a necrotic pathway activated by zVAD-fmk and TNFα, also decrease necrosis in response to a necrotic pathway activated by zVAD-fmk and dimethyl sulfoxide (DMSO).

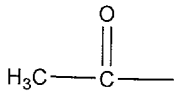
Accordingly, in a first aspect, the invention features a chemical compound in a pharmaceutically acceptable carrier, having the formula:



wherein each R_1 is independently selected from the group consisting of hydrogen, carboxy, methyl, hydroxyl, methoxyl, amino, and nitro; R_2 is selected from the group consisting of hydrogen, alkyl, and acyl; R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl; R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups; X is selected from the group consisting of =O, -OH and -H; Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and each of the bonds (a), (b), and (c) independently is either a double or single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

In a preferred embodiment of the first aspect of the invention, in the compound each R_1 is hydrogen; R_2 and R_3 are each hydrogen; R_4 is a methyl group; X is =O; Y is =S; bond (a) is a double bond; and bonds (b) and (c) are each single bonds.

In another embodiment, the acyl group of R_1 or R_3 is selected from the group consisting of:



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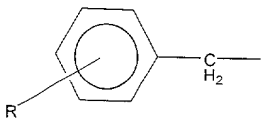
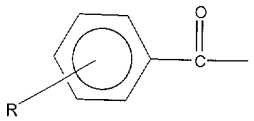


, or



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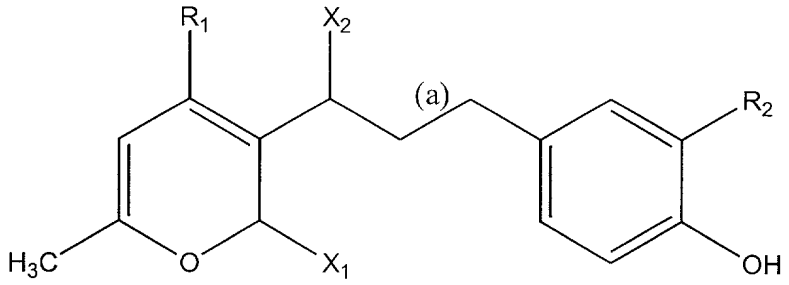
In other embodiments, in the compound if R_1 is a hydrogen, then R_2 and R_3 are not each hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_2 is a hydrogen, then R_1 is a not a hydrogen, or R_3 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_3 is a hydrogen, then R_1 is a not a hydrogen, or R_2 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are

not each single bonds. If R_4 is a methyl group, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In other embodiments, if X is =O, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If Y is =S, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In yet other embodiments, if bond (a) is a double bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bonds (b) and (c) are not each single bonds. If bond (b) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (c) is not a single bond. If bond (c) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (b) is not a single bond.

In a second aspect, the invention features a compound in a pharmaceutically acceptable carrier, having the formula:



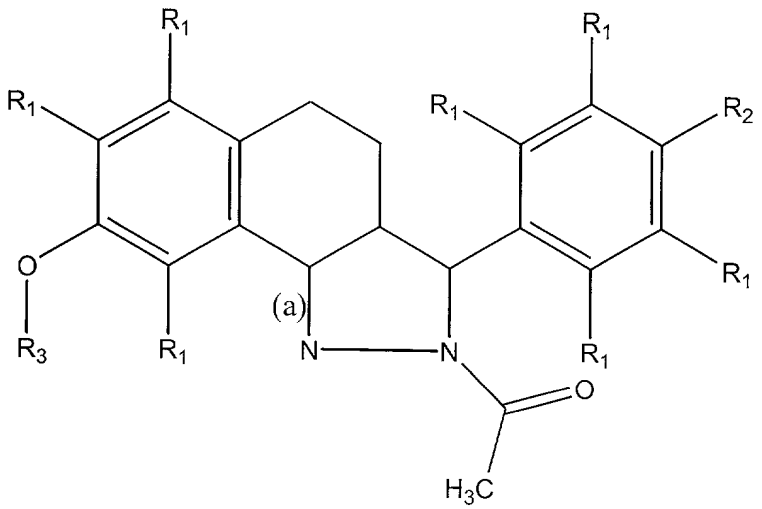
wherein each of X_1 and X_2 is independently selected from the group consisting of =O, -OH and -H; R_1 is selected from the group consisting of hydrogen and hydroxyl; R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and the bond (a) is either a single or double bond.

In a preferred embodiment of the second aspect of the invention, in the compound each of X_1 and X_2 is =O; R_1 is a hydroxyl group; R_2 is a nitro group; and the bond (a) is a double bond.

In other embodiments, if X_1 is =O, then X_2 is not =O; or R_1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If X_2 is =O, then X_1 is not =O; or R_1 is not a hydroxyl group; or R_2 is not a nitro group; or the bond (a) is not a double bond. If R_1 is a hydroxyl group, then each of X_1 and X_2 are not =O; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If R_2 is a nitro group, then each of X_1 and X_2 are not =O; or R_1 is not a hydroxyl group; or the bond (a) is not a double bond. If the bond (a) is a double

bond, then each of X_1 and X_2 are not =O; or R1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond.

In a third aspect, the invention features a chemical compound in a pharmaceutically acceptable carrier, having the formula:



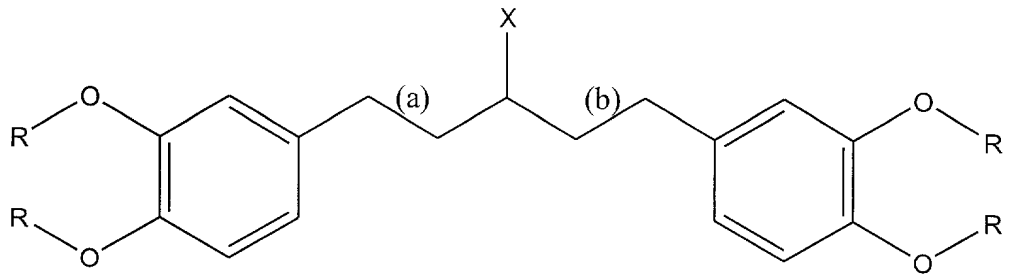
wherein each R_1 and R_2 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl; R_3 is selected from the group consisting of hydrogen and methyl; and the bond (a) is either a single or double bond.

In a preferred embodiment of the third aspect of the invention, in the compound each R_1 is hydrogen; R_2 is fluorine; R_3 is a methyl group; and the bond (a) is a double bond.

In other embodiments of the third aspect of the invention, if R_1 is a hydrogen, then R_2 is not fluorine; or R_3 is not a methyl group; or the bond (a) is not a double bond. If R_2 is a fluorine, then R_1 is not hydrogen; or R_3 is a not a methyl group; or the bond (a) is not a double bond. If R_3 is a methyl group, then R_1 is not

hydrogen, or R_2 is not fluorine; or the bond (a) is not a double bond. If the bond (a) is a double bond, then R_1 is not hydrogen, or R_2 is not fluorine; or R_3 is not a methyl group.

In a fourth aspect, the invention features a chemical compound in a pharmaceutically acceptable carrier, having the formula:

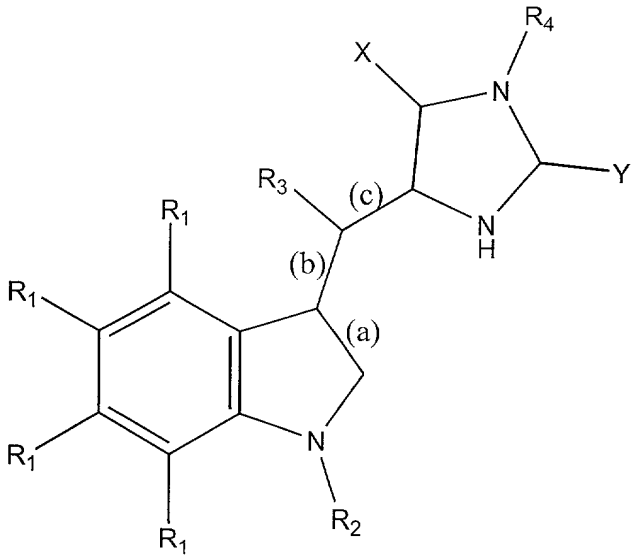


wherein each R is independently selected from the group consisting of H or CH_3; the bond (a) is either a single or double bond; the bond (b) is either a single or double bond; and X is selected from the group consisting of =O, -OH and -H.

In a preferred embodiment of the fourth aspect of the invention, in the compound each R is CH_3; the bonds (a) and (b) are each a double bond; and X is =O.

In other embodiments of the fourth aspect of the invention, if each R is CH_3, then the bonds (a) and (b) are not each a double bond; or X is not =O. If the double bond (a) is a double bond, then each R is not CH_3; or the bond (b) is not a double bond; or X is not =O. If the bond (b) is a double bond, then each R is not CH_3; or the bond (a) is not a double bond; or X is not =O. If X is =O, then R is not CH_3, or the bonds (a) and (b) are not each a double bond.

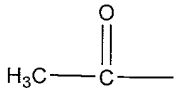
In a fifth aspect, the invention features a method for decreasing necrosis, involving contacting a cell with a chemical compound having the formula:



wherein each R_1 is independently selected from the group consisting of hydrogen, carboxy, methyl, hydroxyl, methoxyl, amino, and nitro; R_2 is selected from the group consisting of hydrogen, alkyl, and acyl; R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl; R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups; X is selected from the group consisting of =O, -OH and -H; Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and each of the bonds (a), (b), and (c) independently is either a double or single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

In a preferred embodiment of the fifth aspect of the invention, in the compound each R_1 is hydrogen; R_2 and R_3 are each hydrogen; R_4 is a methyl group; X is =O; Y is =S; bond (a) is a double bond; and bonds (b) and (c) are each single bonds.

In another embodiment, the acyl group of R_1 or R_3 is selected from the group consisting of:



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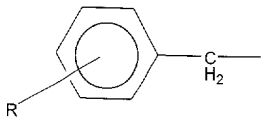

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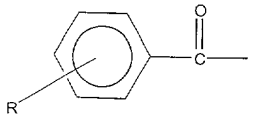


.

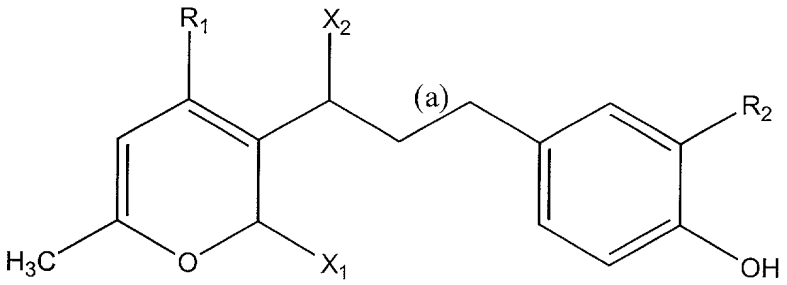
In other embodiments, in the compound if R_1 is a hydrogen, then R_2 and R_3 are not each hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_2 is a hydrogen, then R_1 is a not a hydrogen, or R_3 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_3 is a hydrogen, then R_1 is a not a hydrogen, or R_2 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_4 is a methyl group, then R_1 is a not a hydrogen, or R_2

and R_3 are not each not a hydrogen; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In other embodiments, if X is =O, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If Y is =S, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In yet other embodiments, if bond (a) is a double bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bonds (b) and (c) are not each single bonds. If bond (b) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (c) is not a single bond. If bond (c) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (b) is not a single bond.

In a sixth aspect, the invention features a method for decreasing necrosis, involving contacting a cell with a chemical compound having the formula:

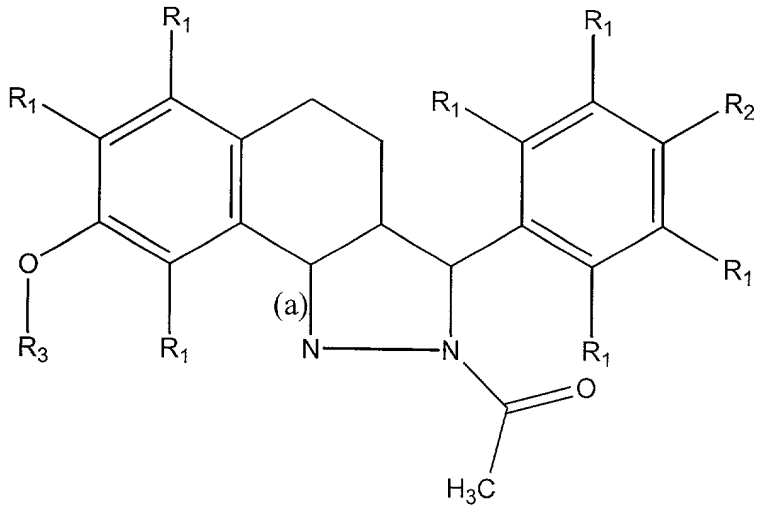


wherein each of X_1 and X_2 is independently selected from the group consisting of =O, -OH and -H; R_1 is selected from the group consisting of hydrogen and a hydroxyl; R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and the bond (a) is either a single or double bond.

In a preferred embodiment of the sixth aspect of the invention, in the compound each of X_1 and X_2 is =O; R_1 is a hydroxyl group; R_2 is a nitro group; and the bond (a) is a double bond.

In other preferred embodiments of the sixth aspect of the invention, if X_1 is =O, then X_2 is not =O; or R_1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If X_2 is =O, then X_1 is not =O; or R_1 is not a hydroxyl group; or R_2 is not a nitro group; or the bond (a) is not a double bond. If R_1 is a hydroxyl group, then each of X_1 and X_2 are not =O; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If R_2 is a nitro group, then each of X_1 and X_2 are not =O; or R_1 is not a hydroxyl group; or the bond (a) is not a double bond. If the bond (a) is a double bond, then each of X_1 and X_2 are not =O; or R1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond.

In a seventh aspect, the invention features a method for decreasing necrosis, involving contacting a cell with a chemical compound having the formula:



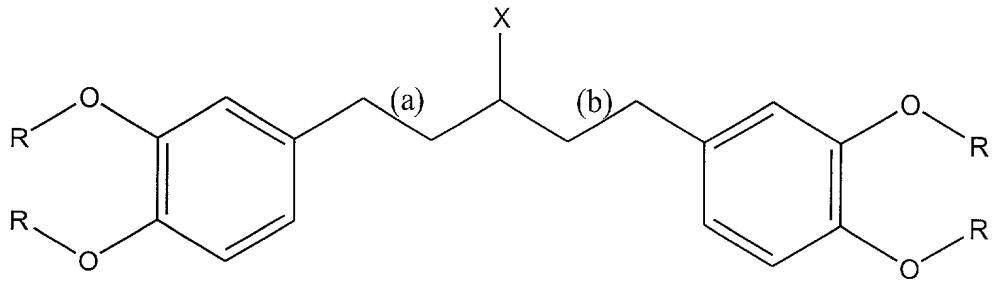
wherein each R_1 and R_2 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl; R_3 is selected from the group consisting of hydrogen and methyl; and the bond (a) is either a single or double bond.

In a preferred embodiment of the seventh aspect of the invention, in the compound each R_1 is hydrogen; R_2 is fluorine; R_3 is a methyl group; and the bond (a) is a double bond.

In other embodiments of the seventh aspect of the invention, if R_1 is a hydrogen, then R_2 is not fluorine; or R_3 is not a methyl group; or the bond (a) is not a double bond. If R_2 is a fluorine, then R_1 is not hydrogen; or R_3 is a not a methyl group; or the bond (a) is not a double bond. If R_3 is a methyl group, then R_1 is not hydrogen, or R_2 is not fluorine; or the bond (a) is not a double bond. If the bond (a) is a double bond, then R_1 is not hydrogen, or R_2 is not fluorine; or R_3 is not a

methyl group.

In an eighth aspect, the invention features a method for decreasing necrosis, involving contacting a cell with a chemical compound having the formula:



wherein each R is independently selected from the group consisting of H or CH_3; the bond (a) is either a single or double bond; the bond (b) is either a single or double bond; and X is selected from the group consisting of =O, -OH and -H.

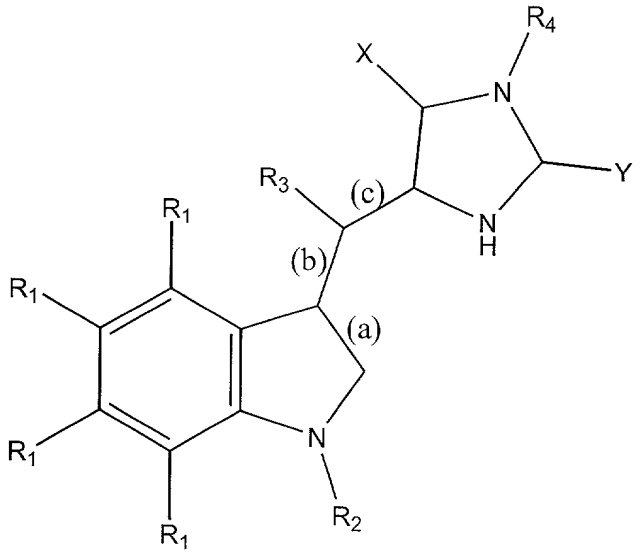
In a preferred embodiment of the eighth aspect of the invention, in the compound each R is CH_3; the (a) and (b) bonds are each a double bond; and X is =O.

In other embodiments of the eighth aspect of the invention, if each R is CH_3, then the bonds (a) and (b) are not each a double bond; or X is not =O. If the double bond (a) is a double bond, then each R is not CH_3; or the bond (b) is not a double bond; or X is not =O. If the bond (b) is a double bond, then each R is not CH_3; or the bond (a) is not a double bond; or X is not =O. If X is =O, then R is not CH_3, or the bonds (a) and (b) are not each a double bond.

In a preferred embodiment of any of the fifth, sixth, seventh, or eighth aspects of the invention, the cell is capable of undergoing necrosis in the presence

of zVAD-fmk and TNFα. In another preferred embodiment, the cell is capable of undergoing necrosis in the presence of zVAD-fmk and DMSO. In yet another preferred embodiment, the cell is mammalian, such as a human or rodent cell. In yet another preferred embodiment, the cell is a neuron. In still another preferred embodiment, the compound is in a pharmaceutically acceptable carrier.

In a ninth aspect, the invention features a method for treating a condition in a subject, involving the steps of administering a chemical compound having the formula:

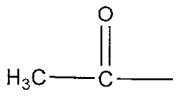


to the subject, in a dosage sufficient to decrease necrosis, wherein each R_1 is independently selected from the group consisting of hydrogen, carboxy, methyl, hydroxyl, methoxyl, amino, and nitro; R_2 is selected from the group consisting of hydrogen, alkyl, and acyl; R_3 is selected from the group consisting of alkyl, acyl,

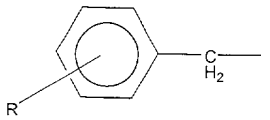
halogen, hydrogen, or hydroxyl; R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups; X is selected from the group consisting of =O, -OH and -H; Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and each of the bonds (a), (b), and (c) independently is either a double or single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

In a preferred embodiment of the ninth aspect of the invention, in the compound each R_1 is hydrogen; R_2 and R_3 are each hydrogen; R_4 is a methyl group; X is =O; Y is =S; bond (a) is a double bond; and bonds (b) and (c) are each single bonds.

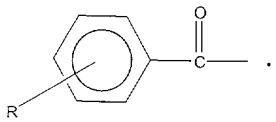
In another embodiment, the acyl group of R_1 or R_3 is selected from the group consisting of:



, or



, or



.

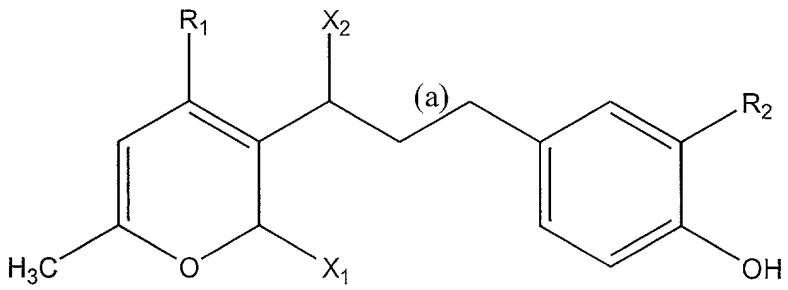
In other embodiments, in the compound if R_1 is a hydrogen, then R_2 and R_3 are not each hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not

=S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_2 is a hydrogen, then R_1 is a not a hydrogen, or R_3 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_3 is a hydrogen, then R_1 is a not a hydrogen, or R_2 is not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If R_4 is a methyl group, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or X is not =O; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In other embodiments, if X is =O, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or Y is not =S; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds. If Y is =S, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or bond (a) is not a double bond; or bonds (b) and (c) are not each single bonds.

In yet other embodiments, if bond (a) is a double bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; or bonds (b) and (c) are not each single bonds. If bond (b) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (c) is not a single bond. If bond (c) is a single bond, then R_1 is a not a hydrogen, or R_2 and R_3 are not each not a hydrogen; or R_4 is not a methyl group; or X is not =O; or Y is not =S; bond (a) is not a double bond or bond (b) is not a single bond.

In a tenth aspect, the invention features a method for treating a condition in a subject, involving the steps of administering a chemical compound having the formula:



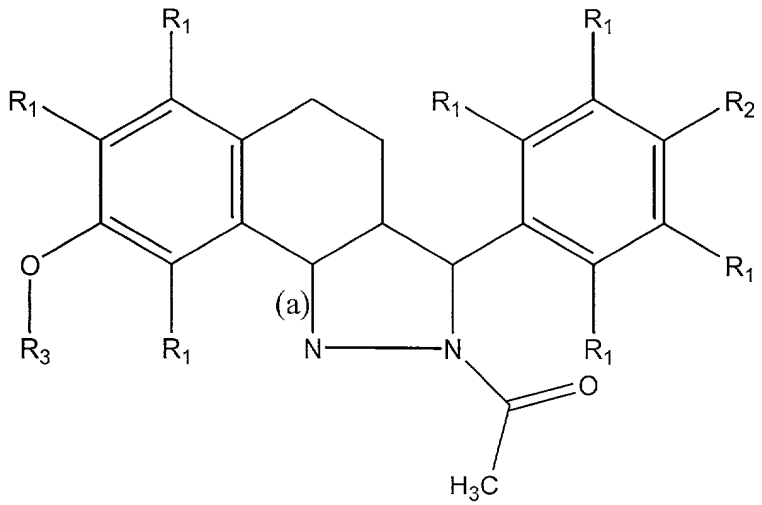
to the subject, in a dosage sufficient to decrease necrosis, wherein each of X_1 and X_2 is independently selected from the group consisting of =O, -OH and -H; R_1 is selected from the group consisting of hydrogen and a hydroxyl; R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and the bond (a) is either a single or double bond.

In a preferred embodiment of the tenth aspect of the invention, in the compound each of X_1 and X_2 is =O; R_1 is a hydroxyl group; R_2 is a nitro group; and the bond (a) is a double bond.

In other embodiments of the tenth aspect of the invention, if X_1 is =O, then X_2 is not =O; or R_1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If X_2 is =O, then X_1 is not =O; or R_1 is not a hydroxyl group; or R_2 is not a nitro group; or the bond (a) is not a double bond. If R_1 is a hydroxyl group, then each of X_1 and X_2 are not =O; or R_2 is a not a nitro group; or the bond (a) is not a double bond. If R_2 is a nitro group, then each of X_1 and X_2 are not =O; or R_1 is not a hydroxyl group; or the bond (a) is not a double

bond. If the bond (a) is a double bond, then each of X_1 and X_2 are not =O; or R1 is not a hydroxyl group; or R_2 is a not a nitro group; or the bond (a) is not a double bond.

In an eleventh aspect, the invention features a method for treating a condition in a subject, involving the steps of administering a chemical compound having the formula:

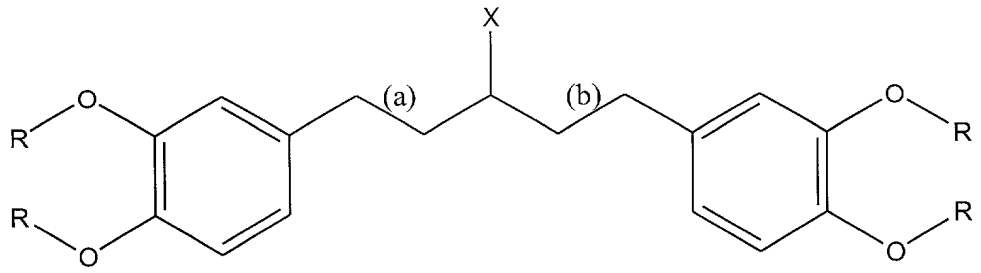


to the subject, in a dosage sufficient to decrease necrosis, wherein each R_1 and R_2 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl; R_3 is selected from the group consisting of hydrogen and methyl; and the bond (a) is either a single or double bond.

In a preferred embodiment of the eleventh aspect of the invention, in the compound each R_1 is hydrogen; R_2 is fluorine; R_3 is a methyl group; and the bond (a) is a double bond.

In other embodiments of the eleventh aspect of the invention, if R_1 is a hydrogen, then R_2 is not fluorine; or R_3 is not a methyl group; or the bond (a) is not a double bond. If R_2 is a fluorine, then R_1 is not hydrogen; or R_3 is a not a methyl group; or the bond (a) is not a double bond. If R_3 is a methyl group, then R_1 is not hydrogen, or R_2 is not fluorine; or the bond (a) is not a double bond. If the bond (a) is a double bond, then R_1 is not hydrogen, or R_2 is not fluorine; or R_3 is not a methyl group.

In a twelfth aspect, the invention features a method for treating a condition in a subject, involving the steps of administering a chemical compound having the formula:



to the subject, in a dosage sufficient to decrease necrosis, wherein each R is independently selected from the group consisting of H or CH_3; the bond (a) is either a single or double bond; the bond (b) is either a single or double bond; and X is selected from the group consisting of =O, -OH and -H.

In a preferred embodiment of the twelfth aspect of the invention, in the compound each R is CH_3; the (a) and (b) bonds are each a double bond; and X is =O.

In other embodiments of the twelfth aspect of the invention, if each R is CH_3, then the bonds (a) and (b) are not each a double bond; or X is not =O. If the double bond (a) is a double bond, then each R is not CH_3; or the bond (b) is not a double bond; or X is not =O. If the bond (b) is a double bond, then each R is not CH_3; or the bond (a) is not a double bond; or X is not =O. If X is =O, then R is not CH_3, or the bonds (a) and (b) are not each a double bond.

In a preferred embodiment of any of the ninth, tenth, eleventh, or twelfth aspects of the invention, the condition is a neurodegenerative disease. Most preferably the neurodegenerative disease is selected from the group consisting of Alzheimer's disease, Huntington's disease, cerebral ischemia, stroke, amyotropic lateral sclerosis, multiple sclerosis, Lewy body disease, Menkes, disease, Wilson disease, Creutzfeldt-Jakob disease, and Fahr disease. In other preferred embodiments, the condition is ischemic brain or heart injury, or head trauma. In another preferred embodiment, the subject is a mammal, such as a human or a rodent.

In a thirteenth aspect, the invention features a method for identifying a compound that decreases necrosis, involving the steps of: providing a cell in which apoptosis is prevented; contacting the cell with a first compound that causes a cell to undergo necrosis; contacting the cell with a second compound; and measuring necrosis relative to a control cell, wherein a decrease in necrosis indicates that the second compound decreases necrosis.

In a preferred embodiment of the thirteenth aspect of the invention, apoptosis is prevented by contacting the cell with zVAD-fmk. In another preferred embodiment, the first compound is TNFα or DMSO.

It will be appreciated that any of the R, X, or Y groups of the compounds of the invention, or of the compounds used in any method of the invention may be alkyl derivatives or contain alkyl linkers.

As used herein, by "decreasing necrosis" is meant reducing the number of cells which undergo necrosis relative to a control cell, receiving a cell death stimulus, such as TNFα/zVAD-fmk or DMSO/zVAD-fmk without a candidate small molecule inhibitor. Preferably necrosis is decreased 10% relative to a control. More preferably necrosis is decreased 50% relative to a control. Most preferably necrosis is decreased 90% relative to a control. Preferably a decrease in necrosis is tested by determining the ATP level in a cell which has received a test compound, such as a compound from a chemical library, and comparing it to the ATP level in a control cell. Necrosis is decreased in a cell treated with a test compound in which the ATP level does not decrease as much as it does in the control cell.

By "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate the level of necrosis by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "cell death" is meant the death of a cell by either apoptosis or necrosis.

As used herein, by "necrosis" is meant caspase-independent cell death characterized by cellular ATP depletion. Preferably the cell is depleted of ATP 10% relative to a control cell, receiving vehicle only (for example, DMSO). More preferably, the cell is depleted of ATP 50% relative to a control cell. Most

preferably, the cell is depleted of ATP 90% relative to a control cell. Preferably, necrosis is tested by determining the ATP level in a cell which has received a compound, for example, zVAD-fmk, DMSO, or TNFα, and comparing it to the ATP level in a cell receiving vehicle only. Necrosis occurs in a cell treated with a test compound in which the ATP level decreases relative to the control cell.

Necrosis may be liquifactive, may affect adipose or hepatic tissue, and may be caseous or fibrinoid. A cell may undergo necrosis in response to ischemic cell injury or viral infection.

By "caspase-independent cell death" is meant cell death that occurs when apoptosis is prevented. Apoptosis may be prevented by contacting a cell with a caspase inhibitor such as zVAD-fmk at a concentration sufficient enough that the cell survives when stimulated to undergo apoptosis, for example, by treatment with an apoptosis-promoting drug or ionizing radiation.

By "apoptosis" is meant cell death characterized by any of the following properties: nuclear condensation, DNA fragmentation, membrane blebbing, or cell shrinkage.

By "modulation of intracellular signaling pathways mediated by TNFα" is meant a change in the communication between components of a cell in response to contacting the cell with TNFα. The change may be in the way or duration in which proteins within the cell interact, or the way or duration in which proteins are altered, such as by phosphorylation or dephosphorylation, or in the way or duration in which proteins interact with DNA.

By "modulation of intracellular signaling pathways mediated by DMSO" is meant a change in the communication between components of a cell in response to contacting the cell with DMSO. The change may be in the way or

duration in which proteins within the cell interact, or the way or duration in which proteins are altered, such as by phosphorylation or dephosphorylation, or in the way or duration in which proteins interact with DNA.

By "treating" is meant to submit or subject an animal, cell, lysate or extract derived from a cell, or a molecule derived from a cell to a test compound that decreases necrosis.

By "condition" is meant a state of being or feeling. Conditions include, but are not limited to, neurodegenerative disease, stroke, liver disease, pancreatic disease, ischemic brain or heart injury or other ischemic injuries, head trauma, a necrotic ulceration, septic shock, coronary heart disease, gastrointestinal disease, tuberculosis, alteration of blood vessels, viral infection (e.g., HIV infection or AIDS), or conditions associated with HIV infection or AIDS.

By "neurodegenerative disease" is meant a disease characterized by neuronal cell death. Examples of neurodegenerative diseases include, but are not limited to, Alzheimer's disease, Huntington's disease and related polyglutamine expansion diseases, cerebral ischemia, stroke, amyotropic lateral sclerosis, multiple sclerosis, Lewy body disease, Menkes disease, Wilson disease, Creutzfeldt-Jakob disease, and Fahr disease.

By "neuron" is meant a cell of ectodermal embryonic origin derived from any part of the nervous system of an animal. Neurons express well-characterized neuron-specific markers which include neurofilament proteins, MAP2, and class III β-tubulin. Included as neurons are, for example, hippocampal, cortical, midbrain dopaminergic, motor, sensory, sympathetic, septal cholinergic, and cerebellar neurons.

By a "dosage sufficient to decrease necrosis" is meant an amount of a chemical compound or small molecule which when administered to a subject will decrease necrosis. Preferably necrosis is decreased in the subject 10% relative to an untreated subject. More preferably necrosis is decreased in the subject 50% relative to an untreated subject. Most preferably necrosis is decreased in the subject 90% relative to an untreated subject.

As used herein, by "measuring necrosis" is meant determining if a cell is dying through necrosis, in the presence of a compound, compared to a cell which is not in the presence of the compound (control cell). Necrosis can be measured by determining cellular ATP levels, wherein a cell that is undergoing necrosis has a decreased level of cellular ATP compared to a control cell. Necrosis may also be measured by staining with a vital dye, for example, trypan blue, wherein a cell which is necrosing will be stained with the vital dye, and a cell which is not necrosing will not be stained with the dye.

By a "derivative" is meant a structural derivative having a chemical modification of the compound which does not reduce the ultimate level of necrosis, but which does enhance bioavailability, solubility, or stability *in vivo* or *ex vivo* or which reduces the toxicity or dosage required. Such modifications are known to those skilled in the field of medicinal chemistry.

The present invention provides a number of advantages. For example, the methods described herein allow for a decrease in cell death occurring through a necrosis pathway. The invention also provides compounds and methods for treating diseases in which necrosis occurs. These compounds and methods can be used to treat conditions such as a neurodegenerative disease, stroke, liver disease, pancreatic disease, ischemic heart or brain injury or other ischemic injuries, head

trauma, septic shock, coronary heart disease, gastrointestinal disease, tuberculosis, alteration of blood vessels, viral infection, such as HIV or AIDS, or conditions associated with a viral infection such as HIV or AIDS.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the chemical structure of a molecule which may be used to decrease necrosis. In this chemical structure each R_1 is independently selected from the group consisting of hydrogen, methyl, carboxy, hydroxyl, methoxyl, amino, and nitro; R_2 is selected from the group consisting of hydrogen, alkyl, and acyl; R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl; R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups; X is selected from the group consisting of =O, -OH and -H; Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and each of the bonds (a), (b), and (c) independently is either a double or single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

Fig. 2 is a schematic representation of the chemical structure of chemical compound ID number 115807 from the ChemBridge chemical compound library.

Fig. 3 is a schematic representation of the chemical structure of a molecule which may be used to decrease necrosis. In this chemical structure, each of X_1 and X_2 is independently selected from the group consisting of =O, -OH and

-H; R_1 is selected from the group consisting of hydrogen and a hydroxyl; R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and the bond (a) is either a single or double bond.

Fig. 4 is a schematic representation of the chemical structure of chemical compound ID number 115681 from the ChemBridge chemical compound library.

Fig. 5 is a schematic representation of the chemical structure of a molecule which may be used to decrease necrosis. In this chemical structure each R_1 and R_2 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl; R_3 is selected from the group consisting of hydrogen and methyl; and the bond (a) is either a single or double bond.

Fig. 6 is a schematic representation of the chemical structure of chemical compound ID number 210227 from the ChemBridge chemical compound library.

Fig. 7 is a schematic representation of the chemical structure of a molecule which may be used to decrease necrosis. In this chemical structure each R is independently selected from the group consisting of H or CH_3; the bond (a) is either a single or double bond; the bond (b) is either a single or double bond; and X is selected from the group consisting of =O, -OH and -H.

Fig. 8 is a schematic representation of the chemical structure of chemical compound ID number 215686 from the ChemBridge chemical compound library.

Detailed Description of the Invention

Described herein are methods for decreasing necrosis, as well as for

treating a condition in a subject. Techniques for carrying out the methods of the invention are now described in detail.

Identification of Chemical Compounds that Decrease Cell Necrosis

Assays that measure cell necrosis may be used to facilitate the identification of molecules that decrease necrosis induced by stimuli, such as zVAD-fmk/TNFα. In one approach, zVAD-fmk is added to the culture media of cells at high density (for example, $5x10^5$ or $7.5x10^5$ cells/ml), which are capable of undergoing necrosis in response to zVAD-fmk/TNFα. Candidate molecules, for example, chemical compounds from the ChemBridge chemical library are added, in varying concentrations to the cells, and the cells are then exposed to TNFα.

The occurrence of necrosis of the treated cells is then measured, for example, by measuring the cellular ATP level of the cells exposed to zVAD-fmk/TNFα (Crouch et al. J. Immunol. Methods 160:81-8, 1993; Storer et al. Mutat. Res. 368:59-101, 1996; and Cree et al. Toxicol. *In Vitro* 11:553-556, 1997). The level of necrosis in the presence of the candidate molecule is compared to the level of necrosis in the absence of the candidate molecule, all other factors (e.g., cell type and culture conditions) being equal. The importance of zVAD-fmk in the invention is to block cell death that may occur by apoptosis, so that cell death by necrosis can be fully unmasked.

In a second approach, a cell may be exposed to a candidate molecule that decreases necrosis at the same time it is exposed to either zVAD-fmk or TNFα. In a third approach, a cell may be exposed to zVAD-fmk and TNFα first, and then to a candidate compound. The level of necrosis that occurs following each of these approaches is measured as described above.

The effect of candidate molecules on necrosis induced by cell death stimuli, for example, zVAD-fmk/TNFα or zVAD-fmk/DMSO, may also be measured by other methods, for example, vital dye staining, using dyes such as trypan blue or acridine orange/ethidium bromide.

Compounds that decrease necrosis may be purified or substantially purified, or may be one component of a mixture of compounds, such as a pool of chemical compounds. In an assay of a mixture of compounds, the occurrence of necrosis is tested against progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to decrease necrosis. A molecule that promotes a decrease in necrosis induced by zVAD-fmk/TNFα is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to decrease necrosis, in a patient with a condition in which necrosis occurs, such as a neurodegenerative disease.

Chemical compounds that are found, by the methods described above, to effectively decrease necrosis induced, for example, by zVAD-fmk/TNFα in an *in vitro* system may be tested further in animal models. Particularly useful animal models include mouse and rat models of cell death, ischemic brain or heart injury or other ischemic injuries, head trauma, neurodegenerative diseases, coronary heart disease, and septic shock. Examples of such models include SOD or Huntington's disease gene transgenic mice, and other known models, such as those described by Li et al. (Hum. Mol. Genet. 8:1227-12236, 1999), Levine et al. (Neurosci. Res. 58:515-532, 1999), Vukosavic et al. (J. Neurochem. 73:2460-2468, 1999), Gruney (J. Neurol. Sci. 152 suppl. 1:S67-73, 1997), Deshmukh et al. (Am. J. Physiol. 273 (4 Pt 1):C1130-1135, 1997), and Isibashi et al. (J. Immunol. 163:5666-5677,

1999). Compounds which demonstrate an ability to decrease necrosis in *in vivo* models may be used as therapeutics to prevent necrosis, as appropriate.

Identification of Chemical Compounds that Decrease zVAD-fmk/DMSO Induced Cell Necrosis

Methods for the identification of chemical compounds that decrease cell necrosis induced, for example, by zVAD-fmk/DMSO at a low cell density (e.g., $1x10^5$ cells/ml) is achieved essentially as described above, except, the inducer of necrosis is zVAD-fmk/DMSO, rather than zVAD-fmk/TNFα.

Structural Derivatives of Chemical Compounds that Decrease Necrosis

The small molecules identified to decrease necrosis may be structurally modified and subsequently used to decrease necrosis, or to treat a subject with a condition in which necrosis occurs. For example, the small molecules may be modified by any of the following processes: reduction of aliphatic double bonds; reduction of aliphatic ketones; substitution of nitro groups with protons, halides, or sulfates; reduction of C=O double bonds in flavone rings; elimination of oxygens attached to flavone rings; substitution of methoxyl groups with hydroxyl groups; attachment of hydroxyl and amino groups to benzyl rings; reduction of C=N double bonds; elimination of a fluoride or its substitution with a hydroxyl or other halide group; substitution of a hydrogen with an alkyl group; introduction of hydroxyl, methoxyl, amino, and nitro groups into the benzyl ring; reduction of the double bond in the position 2 of the indol; introduction of double bonds in the linker between indol and hydantoin moieties; reduction or alkylation of the thiourea moiety; reduction, alkylation, or acylation of the indol amino group;

substitution of the hydantoin 3-methyl group with linear and branching alkyl groups of varying length, and with hydroxyl, methyl, or carboxyl functionalities; and reduction of the hydantoin ketone moiety.

The chemical compounds that decrease necrosis may be modified by one of the above processes or various combinations of the above processes. The methods used to generate structural derivatives of the small molecules that decrease necrosis are readily known to those skilled in the fields of organic and medicinal chemistry.

Therapy

A compound identified as capable of decreasing necrosis, using any of the methods described herein, may be administered to patients or animals with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. The chemical compounds for use in such therapies may be produced and isolated by any standard technique known to those in the field of medicinal chemistry. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the identified compound to patients suffering from a disease in which necrosis occurs. Administration may begin before the patient is symptomatic.

Any appropriate route of administration may be employed. For example, the therapy may be administered either directly to the site of a predicted cell death event (for example, by injection) or systemically (for example, by any conventional administration technique). Administration of the compound may also be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmalic, intraventricular, intracapsular, intraspinal, intracisternal,

intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols. The dosage of the therapeutic compounds in a pharmaceutically-acceptable formulation depends on a number of factors, including the size and health of the individual patient. The dosage to deliver may be determined by one skilled in the art.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" ((19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA). Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for compounds that decreases necrosis include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

The methods and compounds of the present invention may be used to treat a number of diseases, as described above. Such methods and compounds may be particularly useful in treating ischemic brain or heart injury or head trauma. These diseases would be excellent targets of such therapies, as necrosis

occurs in them.

If desired, treatment with a compound identified according to the methods described above, may be combined with more traditional therapies for a disease characterized by cell death, such as tacrine hydrochloride for the treatment of Alzheimer's disease, or interferon β-1a for the treatment of multiple sclerosis.

Preventative Anti-Necrosis Therapy

In a patient diagnosed with a heart disease (e.g., coronary heart disease or ischemic heart injury) or degenerative disease (e.g., a neurodegenerative disease, such as Alzheimer's disease or Huntington's disease), any of the above therapies may be administered before the occurrence of the disease phenotype. In particular, compounds shown to decrease necrosis may be administered by any standard dosage and route of administration (as described above).

The methods of the instant invention may be used to decrease necrosis of a cell or to treat disorders described herein in any mammal, for example, humans, domestic pets, or livestock.

The following examples are provided to illustrate the invention. These examples should not be construed as limiting.

Example 1

Cells Undergo Necrosis in Response to zVAD-fmk and TNFα

The cell lines U-937 and BALB/c 3T3 were assayed for the occurrence of necrosis in response to the combined treatment of zVAD-fmk, a caspase inhibitor, and TNFα, a cell death stimulator. The cells ($5x10^5$ cells/ml) were exposed to zVAD-fmk (100 μM) and human TNFα (40 ng/ml) for 72 hours.

Induction of necrosis was assayed by measuring the cellular ATP levels in response to TNFα (Crouch et al., supra, Storer et al. supra, and Cree et al. supra). Cells which underwent necrosis exhibited decreased cellular ATP levels relative to controls cells which received no treatment, zVAD-fmk (100 μM) alone, or human TNFα (40 ng/ml) alone. It was found that the cells underwent necrosis in response to treatment with zVAD-fmk and TNFα. The cells were also observed morphologically for the occurrence of apoptosis or necrosis, for example, by analyzing the cells for membrane blebbing and nuclear condensation.

Example 2

Identification of Small Molecules that Decrease Necrosis

The U-937 cell line was used to screen a library of 16,000 small molecule chemical compounds for a compound's ability to decrease necrosis induced by exposure of the cell to zVAD-fmk and TNFα. The library of chemical compounds used in this screen were from ChemBridge (ChemBridge Corporation, San Diego CA).

In a primary screen, U-937 cells ($5x10^5$ or $7.5x10^5$ cells/ml) were first exposed to zVAD-fmk (100 μM). Thirty minutes later the same cells were exposed to a chemical compound from the library (5 mg/ml, dissolved in 0.1-0.5 μl of DMSO, giving a final DMSO concentration of 0.3% to 1.5%). After an additional thirty minutes, TNFα (40 ng/ml) was added to the cell culture medium. The cells were then incubated at 37°C for 72 hours, and were then assayed for cellular ATP levels. Compounds which did not prevent a decrease in cellular ATP levels were compounds which did not prevent necrosis in response to treatment of the cell with zVAD-fmk and TNFα. Compounds which maintained cellular ATP

levels were compounds which blocked necrosis triggered by zVAD-fmk and TNFα.

As a result of the primary screen, 50 chemical compounds from the library were identified to decrease necrosis induced by zVAD-fmk and TNFα. These compounds were selected for a second round of screening for compounds that decrease necrosis induced by zVAD-fmk and TNFα.

In a secondary screen, the compounds identified from the first screen, above, to decrease necrosis induced by zVAD-fmk and TNFα were assayed for their potency. Serial dilution of each chemical compound was performed and the compounds were administered to U-937 cells, as per the primary screen. The concentrations of each compound were 70 μM, 23 μM, 8 μM, and 2.5 μM. The level of necrosis occurring in response to zVAD-fmk, TNFα, and the various concentrations of chemical compounds was assayed as described above for the primary screen.

As a result of the secondary screen, four chemical compounds from the ChemBridge library: 115807, 115681, 210227, and 215686 were identified to decrease necrosis in response to exposure of the cell to zVAD-fmk and TNFα.

Example 3

Cells Are Protected From Necrosis Upon Exposure to zVAD-fmk and DMSO

Exposure of low density U-937 cells ($1x10^5$ cells/ml) to zVAD-fmk (100 μM) and DMSO (0.5%) for 72 hours results in cell death by necrosis. The compounds identified to decrease cell necrosis triggered by zVAD-fmk and TNFα, compounds 115807, 115681, 210227, and 215686 from the ChemBridge chemical library, were also evaluated for their ability to decrease cell necrosis induced by zVAD-fmk and DMSO. The cells ($1x10^5$ cells/ml) were first exposed to zVAD-

fmk, and then thirty minutes later to the above-identified small molecules that decrease necrosis. After an additional 30 minutes, the cells were exposed to DMSO. Seventy-two hours after exposure to the compounds, cellular ATP levels were measured, as described above. All four chemical compounds that decreased necrosis induced by zVAD-fmk/ TNFα also decreased necrosis induced by zVAD-fmk/DMSO.

Example 4

The Role of Fas-associated Death Domain Protein in zVAD-fmk/TNFα- or zVAD-fmk/DMSO-induced Necrosis

A cell expressing a dominant negative form of the protein Fas-associated death domain (FADD) can also prevent a cell from undergoing necrosis in response to treatment with zVAD-fmk/TNFα- or zVAD-fmk/DMSO. Jurkat cells were stably transfected with a FADD-FKBP fusion construct (Kawahara A. et al. J. Cell Biol. 143(5):1353-60, 1998). Normally such cells undergo apoptosis when FADD is multimerized. However, these cells, in the presence of the caspase inhibitor zVAD-fmk, are protected from apoptosis, and instead undergo necrosis, thus establishing the dependence of apoptosis in this system on caspase activity and induction of necrosis in the absence of caspases.

The stably transfected Jurkat cells (500,000 cells/ml) were treated with 100 nM of FKBP dimerizer (Arraid Pharmaceuticals; used to stimulate FADD multimerization) in the presence of 100 μM of zVAD-fmk (pre-treated for 1 hour) and compounds from the library identified to decrease necrosis (dissolved in DMSO to give a final DMSO concentration of 0.5 %; added 30 minutes after zVAD-fmk) for 48 hours. Cell viability was then assessed by measuring cellular

ATP levels. The small molecules provided protection from necrosis induced in the presence of zVAD-fmk, but not from apoptosis induced by FADD dimerization in the absence of zVAD-fmk. These results indicate that FADD may be involved in mediating necrosis in response to zVAD-fmk/TNFα- or zVAD-fmk/DMSO. It is possible that the small molecules that decrease necrosis may function by interacting with FADD and disrupting FADD's normal function of promoting necrosis upon treatment of a cell with zVAD-fmk/TNFα- or zVAD-fmk/DMSO.

Example 5

Identification of Intracellular Targets of Small Molecules that Decrease Necrosis

Molecules within a cell that interact with the small molecule compounds that decrease necrosis can be identified using a number of different strategies. Each strategy involves detecting interactions between various proteins from a cell and a small molecule that decreases necrosis, identified according to the methods described above. To identify proteins that interact with a small molecule that decreases necrosis, the small molecule may be bound to a bead, using methods known to those skilled in the art. Each strategy should be carried out using proteins from cells which have been exposed to zVAD-fmk/TNFα- or zVAD-fmk/DMSO.

In one strategy, the signaling complex containing FADD, among other proteins, may be immunoprecipitated, using standard techniques known to those skilled in the art. This complex may then be added to the beads containing the desired small molecule compound that decreases necrosis. Proteins that interact with the small molecule that decreases necrosis may be identified by Western blot

detection of proteins contained in the complex, or other techniques known to those skilled in the field of molecular biology. Any detected binding interactions indicate that the target of the small molecule that decreases necrosis is present in the immunoprecipitated FADD complex.

In a second strategy for identifying targets of a small molecule that decreases necrosis, a cell may be fractionated, and the various fractionated pools may be assayed for interaction with the chemical compound using standard molecular biology techniques. A pool of proteins which interacts with the small molecule that decreases necrosis indicates that the pool contains a protein that is a target of the small molecule that decreases necrosis. The target of the small molecule that decreases necrosis may be isolated using techniques known to those skilled in the art.

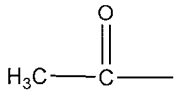
A third strategy involves small pool expression screening systems. Targets of a small molecule that decreases necrosis can be identified from any cell in which the small molecule protects cells from necrosis triggered by zVAD-fmk/TNFα- or zVAD-fmk/DMSO. This method for identifying targets of small molecules that decrease necrosis can be done, for example, according to the methods of Lustig et al. (Methods in Enzymology 283:83-99, 1997). In this method a cDNA library is made from a desired cell line, or any other desired source. The cDNA library is then divided into pools of 100 clones, and the cDNAs are transcribed and translated to form proteins pools for the detection of interactions between a protein and a small molecule that decreases necrosis. Interactions between the small molecules that decrease necrosis and pools of library proteins can be detected using standard molecular biology techniques, for example, SDS-PAGE.

Example 6

Structural Derivatives of Small Molecules that Decrease Necrosis

The following modifications of the small molecules that decrease necrosis may be made and evaluated for their efficacy in decreasing necrosis, for example, that induced by zVAD-fmk/TNFα or zVAD-fmk/DMSO.

The chemical compound 115807 may be modified by the introduction of a hydroxyl, methyl, carboxy, methoxyl, amino or nitro group into the benzyl ring (for example, at any or all of the R_1 positions of Fig. 1). Double bonds may be introduced in the linker between indol and hydantoin moieties (for example, in Fig. 1, bonds (a), (b), or (c) may be double bonds, provided that not both bonds (a) and (b) are double bonds). The thiourea moiety may be reduced or alkylated (for example, the moiety may be -SH or SR_5, wherein R_5 is an alkyl group). The indol amino group may be reduced, alkylated, or acylated (for example, in Fig. 1, R_2 may be CH3, $CH_3(CH_2)_n$, where n is between 1 and 4 and, $HOOC\text{-}(CH_2)_n$, where n is between 1 and 4,



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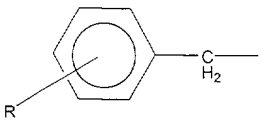

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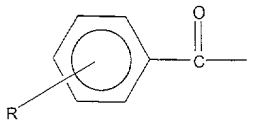
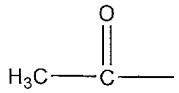


.

In addition, the hydantoin 3-methyl group may be substituted with linear or branching alkyl groups of varying length, and with hydroxyl, methyl, or acyl functionalities (for example the following groups may be present at the R4 position of Fig. 1; CH_3, $CH_3(CH_2)_n$ where n is between 1 and 4, OH, or HOOC-$(CH_2)_n$-, wherein n is between 1 and 4. In addition, the linker CH_2 group between the indol and hydantoin moieties can be alkylated, acylated, halogenated, or hydroxylated (for example, in Fig. 1, R_4 may be CH_3, $CH_3(CH_2)_n$, where n is between 1 and 4, HOOC-$(CH_2)_n$, where n is between 1 and 4,



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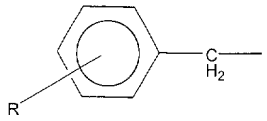

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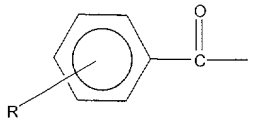


.

Lastly, the hydantoin ketone moiety may be reduced to a hydroxyl group or a hydrogen.

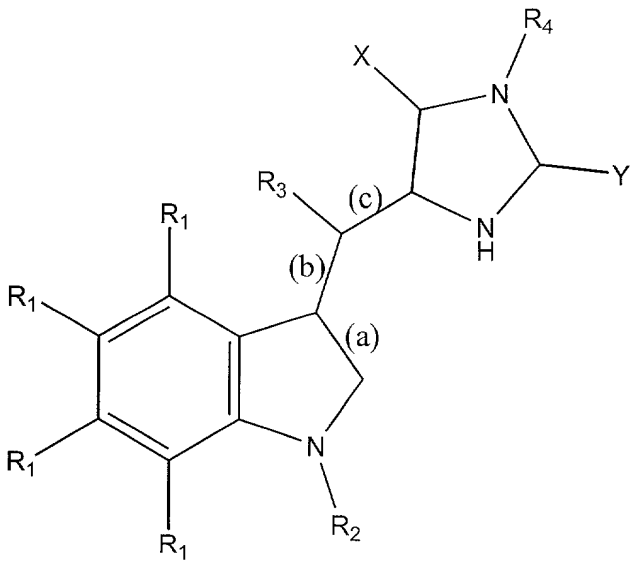
The chemical compound 210227 may be modified by the attachment of a halide, or hydroxyl or amino groups to either or both of the benzyl rings (for example, in the R_1 or R_2 positions of Fig. 5). The C=N double bond may be reduced, or the fluoride may be eliminated or substituted with a hydroxyl group or other halide.

The chemical compound 215686 may be modified by reducing the two central aliphatic double bonds, together, or each one individually. The ketone may also be reduced, or the methoxyl groups may be substituted with hydroxyl groups, each individually, or together.

The chemical compound 115681 may be modified in the following ways. The aliphatic double bond or the aliphatic ketone may be may be reduced. The nitro group may be substituted with a proton, halide, or sulfate. The C=O double bond in the flavone ring may be reduced. Either one or two of the oxygens attached to the flavone may also be eliminated.

What is claimed is:

1. A chemical compound in a pharmaceutically acceptable carrier, said compound having the formula:



wherein

each R_1 is independently selected from the group consisting of hydrogen, methyl, carboxy, hydroxyl, methoxyl, amino, and nitro;

R_2 is selected from the group consisting of hydrogen, alkyl, and acyl;

R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl;

R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups;

X is selected from the group consisting of =O, -OH and -H;

Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and

each of the bonds (a), (b), and (c) independently is either a double or

single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

2. The compound of claim 1, wherein

each R_1 is hydrogen;

R_2 and R_3 are each hydrogen;

R_4 is a methyl group;

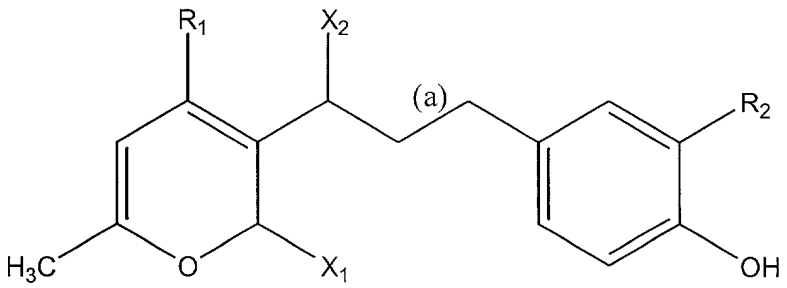
X is =O;

Y is =S;

bond (a) is a double bond; and

bonds (b) and (c) are each single bonds.

3. A compound in a pharmaceutically acceptable carrier, said compound having the formula:



wherein

each of X_1 and X_2 is independently selected from the group consisting of =O,

-OH and -H;

R_1 is selected from the group consisting of hydrogen and a hydroxyl;

R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and

the bond (a) is either a single or double bond.

4. The compound of claim 3, wherein

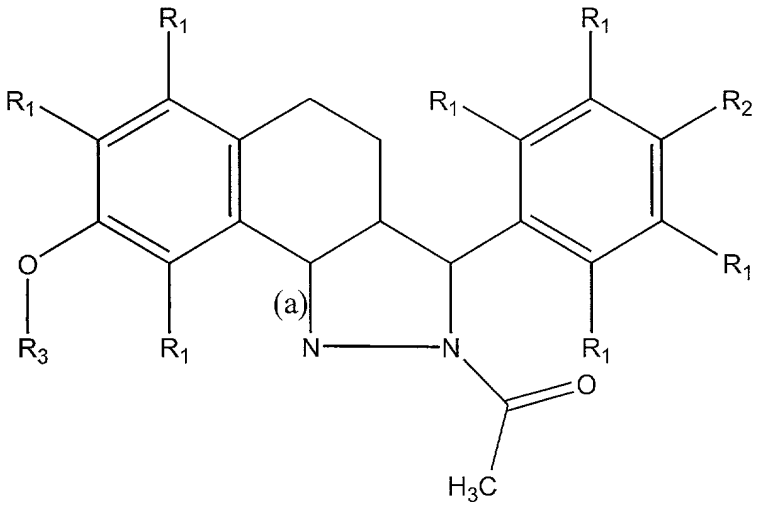
each of X_1 and X_2 is =O;

R_1 is a hydroxyl group;

R_2 is a nitro group; and

the bond (a) is a double bond.

5. A chemical compound in a pharmaceutically acceptable carrier, said compound having the formula:



wherein

each R_1 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl;

R_2 is selected from the group consisting of hydrogen, halide, and hydroxyl;

R_3 is selected from the group consisting of hydrogen and methyl; and

the bond (a) is either a single or double bond.

6. The compound of claim 5, wherein

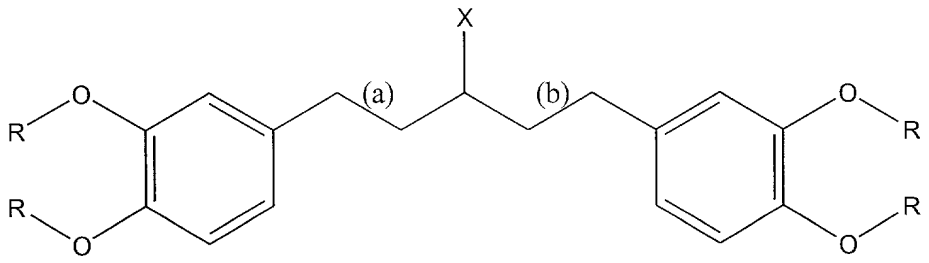
each R_1 is hydrogen;

R_2 is fluorine;

R_3 is a methyl group; and

the bond (a) is a double bond.

7. A chemical compound in a pharmaceutically acceptable carrier, said compound having the formula:



wherein

each R is independently selected from the group consisting of H or CH_3;

the bond (a) is either a single or double bond;

the bond (b) is either a single or double bond; and

X is selected from the group consisting of =O, -OH and -H.

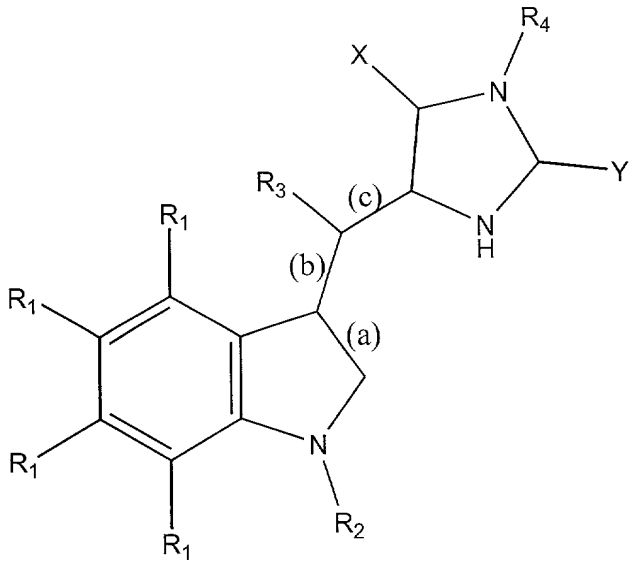
8. The compound of claim 7, wherein

each R is CH_3;

the (a) and (b) bonds are each a double bond; and

X is =O.

9. A method for decreasing necrosis, said method comprising contacting a cell with a chemical compound, said compound having the formula:



wherein

each R_1 is independently selected from the group consisting of hydrogen, methyl, carboxy, hydroxyl, methoxyl, amino, and nitro;

R_2 is selected from the group consisting of hydrogen, alkyl, and acyl;

R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl;

R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups;

X is selected from the group consisting of =O, -OH and -H;

Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and

each of the bonds (a), (b), and (c) independently is either a double or

single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

10. The method of claim 9, wherein in said compound

each R_1 is hydrogen;

R_2 and R_3 are each hydrogen;

R_4 is a methyl group;

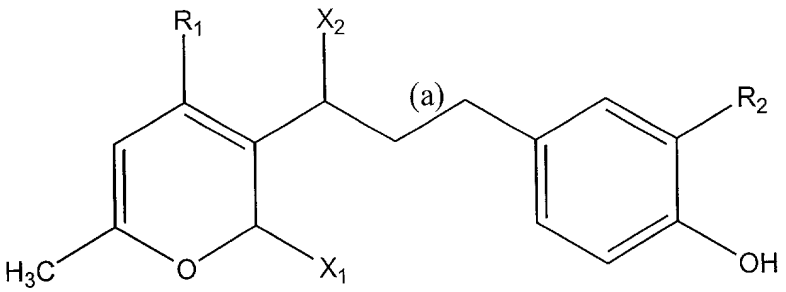
X is =O;

Y is =S;

bond (a) is a double bond; and

bonds (b) and (c) are each single bonds.

11. A method for decreasing necrosis, said method comprising contacting a cell with a chemical compound having the formula:



wherein

each of X_1 and X_2 is independently selected from the group consisting of =O,

-OH and -H;

R_1 is selected from the group consisting of hydrogen and a hydroxyl;

R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and

the bond (a) is either a single or double bond.

12. The method of claim 11, wherein in said compound,

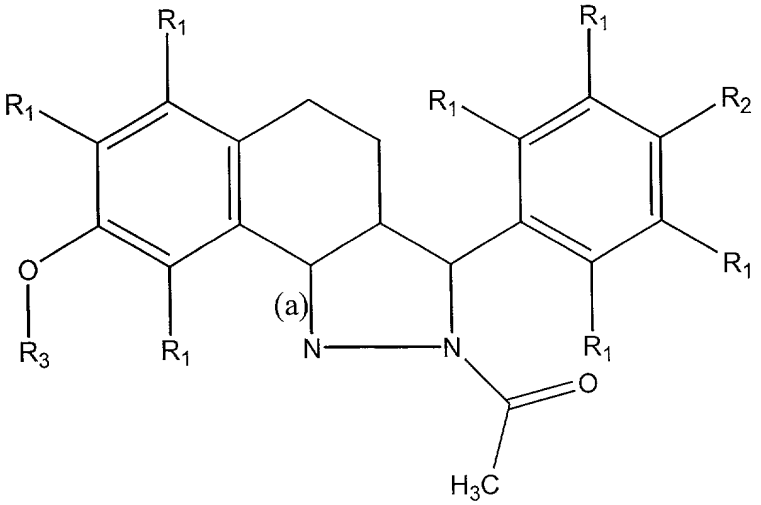
each of X_1 and X_2 is =O;

R_1 is a hydroxyl group;

R_2 is a nitro group; and

the bond (a) is a double bond.

13. A method for decreasing necrosis, said method comprising contacting a cell with a chemical compound having the formula:



wherein

each R_1 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl;

R_2 is selected from the group consisting of hydrogen, halide, and hydroxyl;

R_3 is selected from the group consisting of hydrogen and methyl; and

the bond (a) is either a single or double bond.

14. The method of claim 13, wherein in said compound

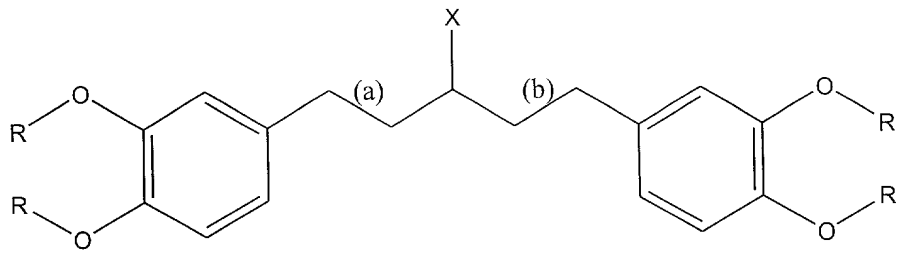
each R_1 is hydrogen;

R_2 is fluorine;

R_3 is a methyl group; and

the bond (a) is a double bond.

15. A method for decreasing necrosis, said method comprising contacting a cell with a chemical compound having the formula:



wherein

each R is independently selected from the group consisting of H or CH_3;

the bond (a) is either a single or double bond;

the bond (b) is either a single or double bond; and

X is selected from the group consisting of =O, -OH and -H.

16. The method of claim 15, wherein in said compound

each R is CH_3;

the (a) and (b) bonds are each a double bond; and

X is =O.

17. The method of any of claims 9, 11, 13, or 15, wherein said cell is capable of undergoing necrosis in the presence of zVAD-fmk and TNFα.

18. The method of any of claims 9, 11, 13, or 15, wherein said cell is capable of undergoing necrosis in the presence of zVAD-fmk and DMSO.

19. The method of any of claims 9, 11, 13, or 15, wherein said cell is mammalian.

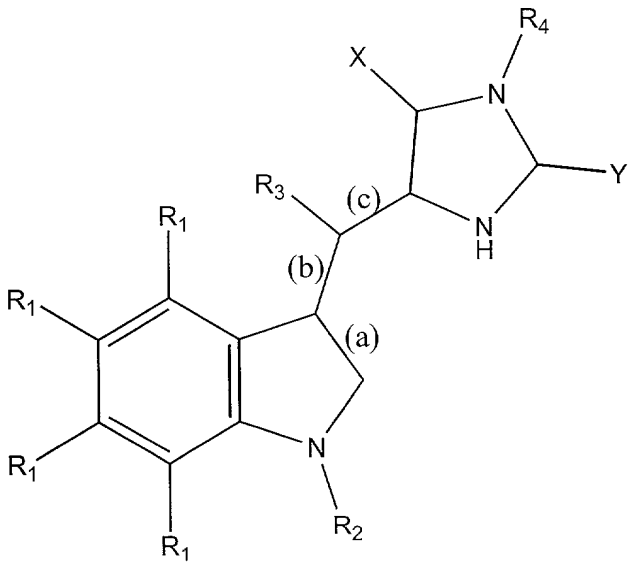
20. The method of claim 19, wherein said cell is human.

21. The method of claim 19, wherein said cell is a neuron.

22. The method of claim 19, wherein said cell is a rodent cell.

23. The method of any of claims 9, 11, 13, or 15, wherein said compound is in a pharmaceutically acceptable carrier.

24. A method for treating a condition in a subject, said method comprising the steps of administering a chemical compound having the formula:



to said subject, in a dosage sufficient to decrease necrosis, wherein

each R_1 is independently selected from the group consisting of hydrogen, methyl, carboxy, hydroxyl, methoxyl, amino, and nitro;

R_2 is selected from the group consisting of hydrogen, alkyl, and acyl;

R_3 is selected from the group consisting of alkyl, acyl, halogen, hydrogen, or hydroxyl;

R_4 is selected from the group consisting of methyl, hydroxyl, carboxyl, and linear and branching alkyl groups;

X is selected from the group consisting of =O, -OH and -H;

Y is selected from the group consisting of =S and $-SR_5$, where R_5 is either hydrogen or an alkyl group; and

each of the bonds (a), (b), and (c) independently is either a double or single bond, provided, however, that bond (a) and bond (b) are not both double bonds.

25. The method of claim 24, wherein in said compound

each R_1 is hydrogen;

R_2 and R_3 are each hydrogen;

R_4 is a methyl group;

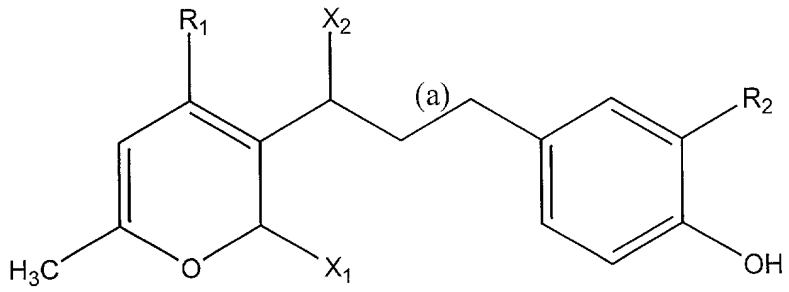
X is =O;

Y is =S;

bond (a) is a double bond; and

bonds (b) and (c) are each single bonds.

26. A method for treating a condition in a subject, said method comprising the steps of administering a chemical compound having the formula:



to said subject, in a dosage sufficient to decrease necrosis wherein

each of X_1 and X_2 is independently selected from the group consisting of =O,

-OH and -H;

R_1 is selected from the group consisting of hydrogen and a hydroxyl;

R_2 is selected from the group consisting of hydrogen, sulfate, nitro, and halide; and

the bond (a) is either a single or double bond.

27. The method of claim 26, wherein in said compound

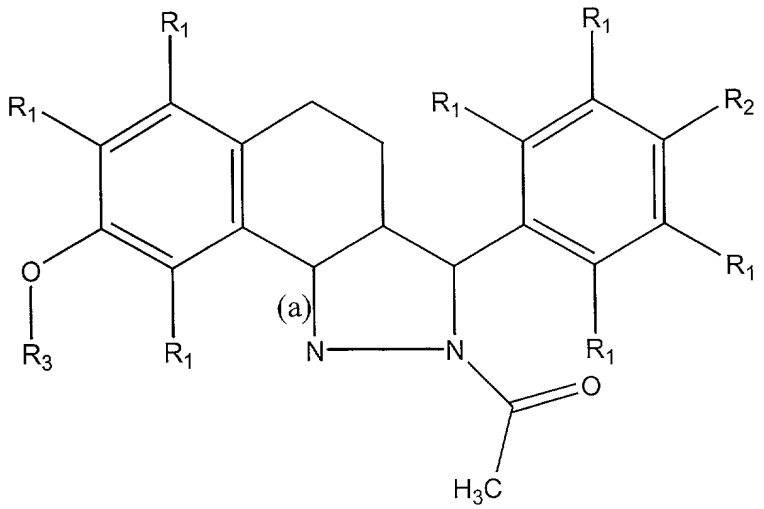
each of X_1 and X_2 is =O;

R_1 is a hydroxyl group;

R_2 is a nitro group; and

the bond (a) is a double bond.

28. A method for treating a condition in a subject, said method comprising the steps of administering a chemical compound having the formula:



to said subject, in a dosage sufficient to decrease necrosis wherein

each R_1 is independently selected from the group consisting of hydrogen, amino, halide, and hydroxyl;

R_2 is selected from the group consisting of hydrogen, halide, and hydroxyl;

R_3 is selected from the group consisting of hydrogen and methyl; and

the bond (a) is either a single or double bond.

29. The compound of claim 28, wherein in said compound

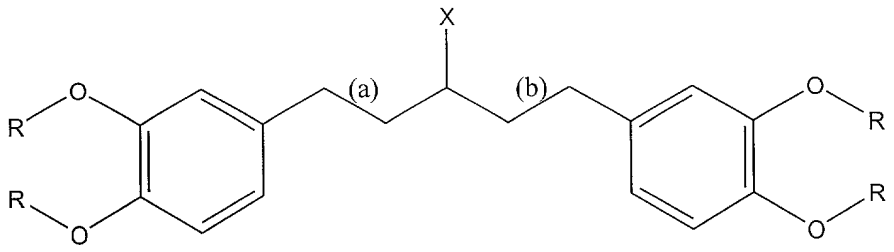
each R_1 is hydrogen;

R_2 is fluorine;

R_3 is a methyl group; and

the bond (a) is a double bond.

30. A method for treating a condition in a subject, said method comprising the steps of administering a chemical compound having the formula:



to said subject, in a dosage sufficient to decrease necrosis wherein

each R is independently selected from the group consisting of H or CH_3;

the bond (a) is either a single or double bond;

the bond (b) is either a single or double bond; and

X is selected from the group consisting of =O, -OH and -H.

31. The compound of claim 30, wherein in said compound

each R is CH_3;

the (a) and (b) bonds are each a double bond; and

X is =O.

32. The method of any of claims 24, 26, 28, or 30, wherein said condition is a neurodegenerative disease.

33. The method of claim 32, wherein said neurodegenerative disease is selected from the group consisting of Alzheimer's disease, Huntington's disease,

cerebral ischemia, stroke, amyotropic lateral sclerosis, multiple sclerosis, Lewy body disease, Menkes, disease, Wilson disease, Creutzfeldt-Jakob disease, and Fahr disease.

34. The method of any of claims 24, 26, 28, or 30, wherein said condition is selected from the group consisting of ischemic brain injury, ischemic heart injury, and head trauma.

35. The method of any of claims 24, 26, 28, or 30, wherein said subject is a mammal.

36. The method of claim 35, wherein said subject is a human.

37. The method of claim 35, wherein said subject is a rodent.

38. A method for identifying a compound that decreases necrosis, comprising the steps of :

(a) providing a cell in which apoptosis is prevented;

(b) contacting said cell with a first compound that causes a cell to undergo necrosis;

(c) contacting said cell with a second compound; and

(d) measuring necrosis relative to a control cell,

wherein a decrease in necrosis indicates that said second compound decreases necrosis.

39. The method of claim 38, wherein said apoptosis is prevented by contacting said cell with zVAD-fmk.

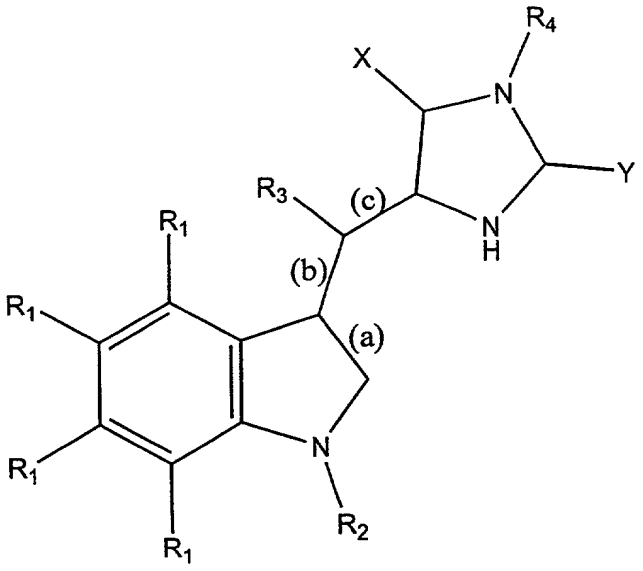
40. The method of claim 38, wherein said first compound is TNFα or DMSO.

SMALL MOLECULE INHIBITORS OF NECROSIS

Abstract of the Disclosure

The invention features methods for decreasing necrosis. The invention also features methods for treating a subject with a condition in which necrosis occurs. The invention further features chemical compounds used to decrease necrosis.

Fig. 1



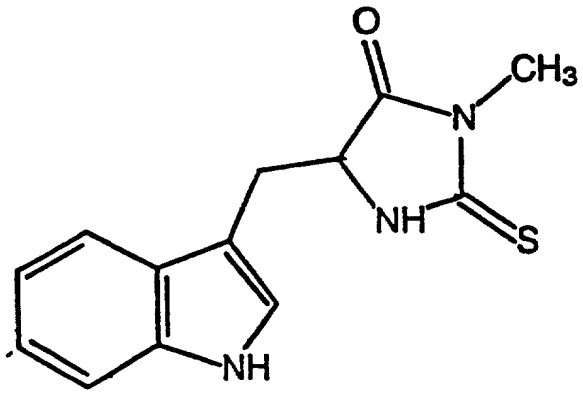
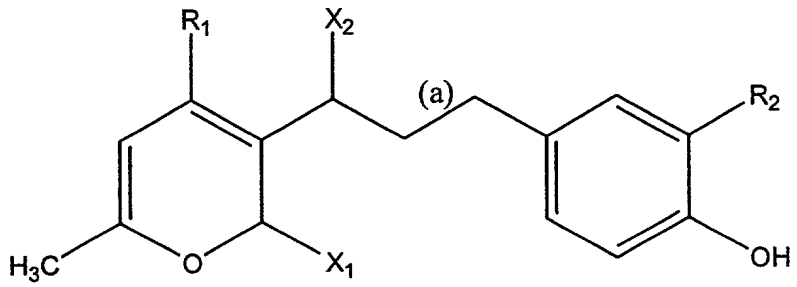


Fig. 2

Fig. 3



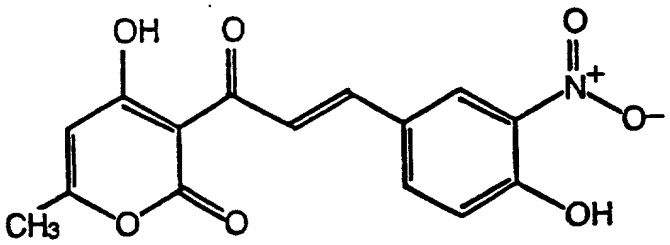
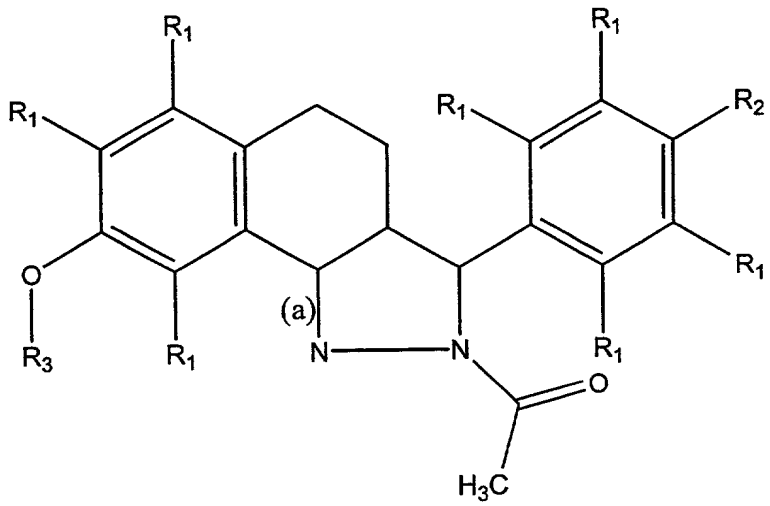


Fig. 4

Fig. 5



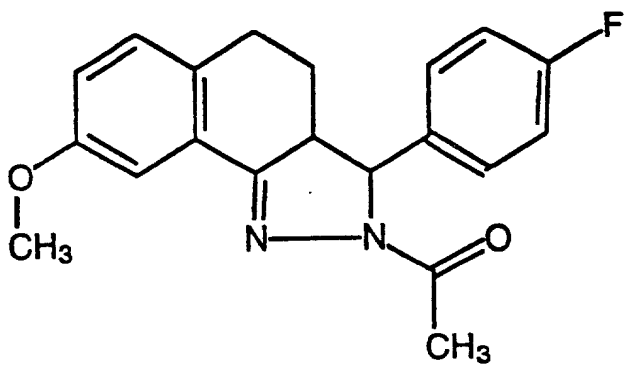
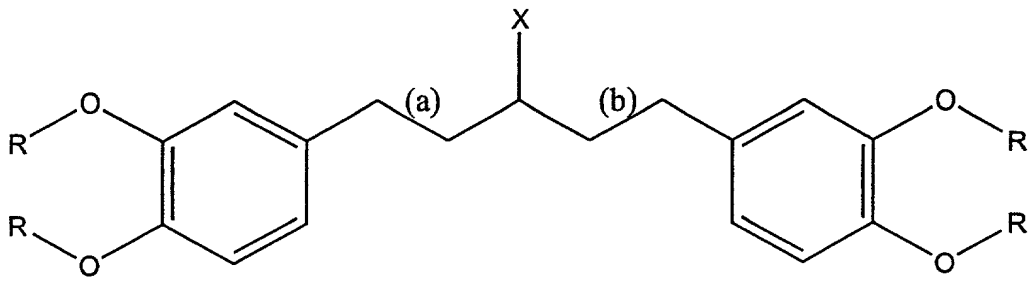


Fig. 6

Fig. 7



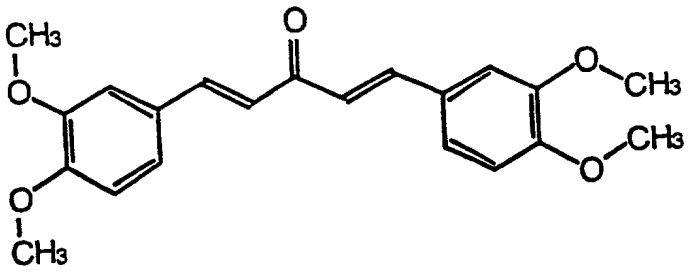


Fig. 8

PATENT
ATTORNEY DOCKET NO: 00742/056003

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled SMALL MOLECULE INHIBITORS OF NECROSIS, the specification of which

- ■ is attached hereto.
- ☐ was filed on _____ as Application Serial No. _____ and was amended on _____.
- ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

FOREIGN PRIORITY RIGHTS: I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
			Yes/No

PROVISIONAL PRIORITY RIGHTS: I hereby claim priority benefits under Title 35, United States Code, §119(e) and §120 of any United States provisional patent application(s) listed below filed by an inventor or inventors on the same subject matter as the present application and having a filing date before that of the application(s) of which priority is claimed:

Serial Number	Filing Date	Status
60/159,668	10/15/99	Pending
60/174,749	01/06/00	Pending

NON-PROVISIONAL PRIORITY RIGHTS: I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the

COMBINED DECLARATION AND POWER OF ATTORNEY

claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Filing Date	Status

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885, Mary Rose Scozzafava, Ph.D., Reg. No.36,268, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506, Timothy J. Douros, Reg. No. 41,716.

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Address all correspondence to: Kristina Bieker-Brady, Ph.D. at Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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COMBINED DECLARATION AND POWER OF ATTORNEY

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Signature:			Date: